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14. ABSTRACT

We hypothesized that the NF1 protein Neurofibromin has functions in addition to its Ras-GAP activity and proposed to develop a yeast NF1 model to define new functions as well as novel regulators of known functions. In year 1 of this project, we categorized genetic hits that we recovered from yeast screens. We divided hits into Ras-dependent and Ras-independent categories. To do this, we used two approaches. First, we assayed mutant yeast strains from our screens by idodine staining, providing an indirect readout for ras signaling levels. For strains that showed elevated ras signaling, we next used Ras-GTP pull-down assays to define if the genes functioned upstream or downstream of ras. The most significant finding during the first year of this project is that we defined which of our genes that interact with *IRA1* or *IRA2* are involved in ras signaling and which are not. Furthermore, of the genes involved in ras signaling, we now have evidence placing these either upstream or downstream of ras. These data will facilitate future years of funding for this project, as we define if the genetic modifiers interact physically with Ira1 and/or Ira2, as well as generating transgenic flies to validate our results in Drosophila.

15. SUBJECT TERMS
NF1, yeast, IRA1, IRA2, ras, GTPase, pull-down, signaling, genetic screen, iodine staining, neurofibromin, GTPase-activating protein

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Annual Report Text

Introduction

Type 1 Neurofibromatosis (NF1) is a devastating human cancer syndrome, characterized by benign and malignant tumors of primarily neural crest origin. NF1 is caused by mutations in the *NF1* gene, which encodes a large protein, called Neurofibromin. Neurofibromin is a large protein and has been shown to be able to function as a Ras-GTPase-activating protein (Ras GAP) to down-regulate ras signaling. Accordingly, NF1-defiicent tumors show elevated ras signaling levels. Defining novel regulators of Neurofibromin's function will help to suggest therapeutic interventions. Because the Ras-GAP domain only comprises a small portion of the protein, we hypothesize that Neurofibromin has cellular functions in addition to its Ras-GAP activity. We have developed a yeast NF1 model to define NF1 disease mechanisms. Budding yeast, *Saccharomyces cerevisiae*, have two NF1-like genes, called *IRA1* and *IRA2*. We generated *ira1Δ* and *ira2Δ* mutant cells and used these to perform genomewide genetic screens to identify, in an unbiased fashion, genes and pathways that interact functionally with Ira1 and Ira2 in yeast, and hopefully Neurofibromin in mammalian cells.

The main goal of this project is to define new functions for Neurofibromin. We have in hand a set of yeast genes that we discovered by using genetic screens to identify synthetic genetic interactors. These included aggravating (worse phenotype) as well as alleviating (better phenotype) genetic interactions. Some of these genes might interact with Ira1 and/or Ira2 in a ras-depdendent or –independent manner. Moreover, for the genes that are involved in the ras pathway, this might be upstream or downstream of ras. The goal of year 1 of this project was to categorize our screen hits based on their effects on the ras pathway. As detailed below, we have used two approaches (iodine staining and Ras-GTP activity assays) to assess the effect of each hit on ras signaling. These data will facilitate year 2 of this project as we continue to define the mechanism of action of these novel Ira1- and Ira2-interacting genes.

Body

We have established a yeast model of neurofibromin 1 (NF1) by employing the yeast *NF1* homologs, *IRA1* and *IRA2*, to conduct Synthetic Genetic Array (SGA) analysis, to dissect the function and signaling pathway of NF1. Using *ira1*Δ and *ira2*Δ mutants as query strains, we have finished multiple rounds of SGA analysis on the yeast deletion library (~4,850 different strains) and multiple rounds of SGA analysis on the new yeast TS (temperature-sensitive) library, which allows us to also survey essential genes (previously not possible).

After each round of screening, the size of each colony on the plate was analyzed by image analysis software, normalized to eliminate edge effects and other growth differences. At least three different replicates of the same screen results were combined together to get the average size and variation of each colony. The positive hits that had a synthetic genetic interaction with the query strain were selected based on the ratio of double mutant to single mutant (>=1.5 or <=0.5), the size different of double and single mutant (>=0.2 or <=-0.2), and the T score (>=7 or <= -7). Only hits that met all three standards were picked as positive hits. For *ira1*Δ, we identified 118 hits from deletion library screen (97 synthetic sick interactions, 21 synthetic alleviating interactions) and 138 hits from the TS library screen (90 synthetic sick interactions from 61 TS mutants, 48 synthetic alleviating interactions from 43 TS mutants).

For *ira2*Δ, we identified 135 hits from deletion library screen (95 synthetic sick interactions, 40 synthetic alleviating interactions) and 116 hits from the TS library screen (29 synthetic sick interactions from 23 TS mutants, 87 synthetic alleviating interactions from 68 TS mutants).

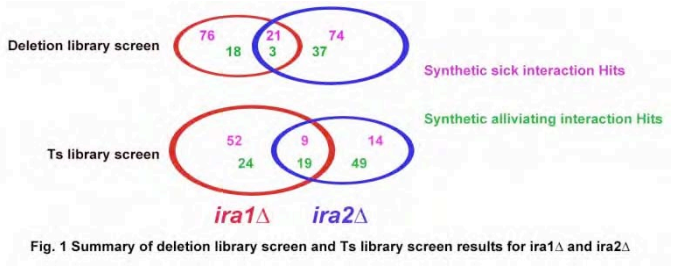

Fig. 1 Summary of deletion library screen and Ts library screen results for ira1Δ and ira2Δ

Interestingly, there were only 24 overlapping hits between ira1 and ira2 deletion library screen, and 28 unique genes overlapping hits between ira1 and ira2 TS library screen (Fig. 1). These data strongly suggest that *IRA1* and *IRA2* are only partially redundant and might have different functions and be involved in distinct signaling pathways.

As proof of principle that our approach was working, from the deletion library screens, we recovered multiple genes already known to be involved the RAS signaling pathway, such as *GPB1, GPR1, PDE2, RAS2, WSC3* as hits. Similarly, for the TS library screen, multiple alleles of the same gene gave similar results; the same mutant at different positions in the library all showed up to have same type of genetic interaction with the query strain, strongly suggesting that the screen is capable of identifying very reliable and reproducible genetic interactions.

One of the well defined phenotypes of *ira1*Δ and *ira2*Δ mutants is the elevated Ras-GTP (active RAS form) content, which can be revealed by Ras-GTP pull-down assay using the Ras-GTP specific binding protein Raf1-RBD conjugated to GST beads. However, it is very time-consuming and not cost-efficient to analyze the Ras-GTP content for so many hits by pull-down assay. To overcome this obstacle, we turned to iodine staining. It has been reported that domain-active Ras mutants have defects in glycogen storage, which can be detected by iodine staining.

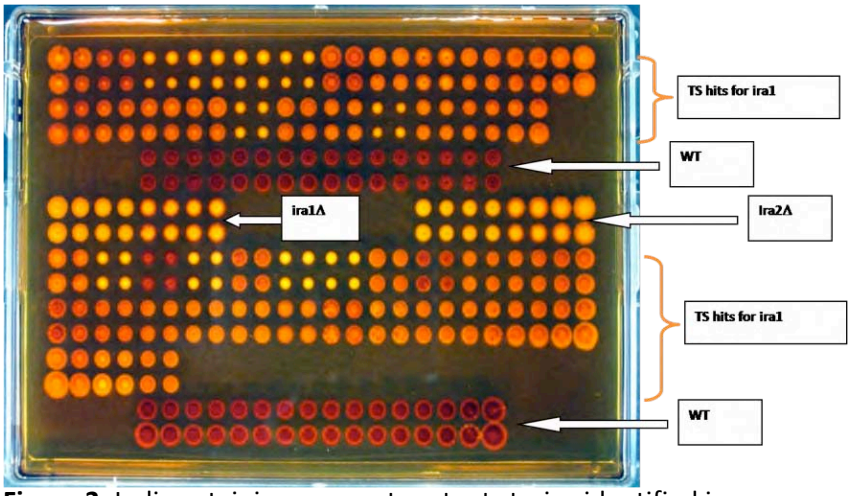

Figure 2. Iodine staining on yeast mutant strains identified in screens with NF1 homologs *ira1*Δ and *ira2*Δ. Red color of the WT strain indicates proper glycogen storage whereas the lighter color indicates a ras-dependent glycogen storage defect.

We reasoned that we could rapidly screen through out hits by iodine staining, to quickly sort them into ras-dependent and ras-independent groups. As shown in Fig. 2, among 118 hits from the deletion library screen for *ira1*Δ, 38 hits were stained lighter than WT by iodine, indicating a glycogen storage defect. For *ira2*Δ, 36 out of 135 deletion library screen hits displayed lighter iodine staining than WT. From the TS library screen hits, 23 hits of *ira1*Δ and 27 hits of *ira2*Δ showed lighter iodine staining than WT. Thus, these hits are most likely involved in the Ras signaling pathway and will be the best candidates for Ras-GTP activity assays. The other hits likely interact with Ira1 and/or Ira2 in a ras-independent manner.

To further define the position of these iodine staining hits in the Ras signaling pathway, we performed Ras-GTP-pull-down assays to analyze the Ras-GTP content in these mutant strains. The original Ras-pull down assay kit was developed for mammalian cells. We have modified the procedure so that it can be applied to yeast Ras and the protein sample concentration can be normalized, therefore the Ras-GTP content can be effectively compared between different strains. As expected, the Ras pull-down assay is very sensitive to GTP hydrolysis and therefore not suitable to analyze large numbers of samples at one time. Indeed, this was the major motivation for us to use iodine staining as an initial way to triage hits. So far, we have completed Ras-GTP pull-down assays for 24 of the strongest hits from our screens. Among these hits, we have found *wsc3Δ, act1-119, act1-136, cdc24-2, cdc24-5, and arp3-31 pre2-75, taf4-18, rpt6-1, rpt6-20*, and *sfh1-1* have higher Ras-GTP content (Fig. 3). We are currently repeating the pull-down assays to confirm these results.

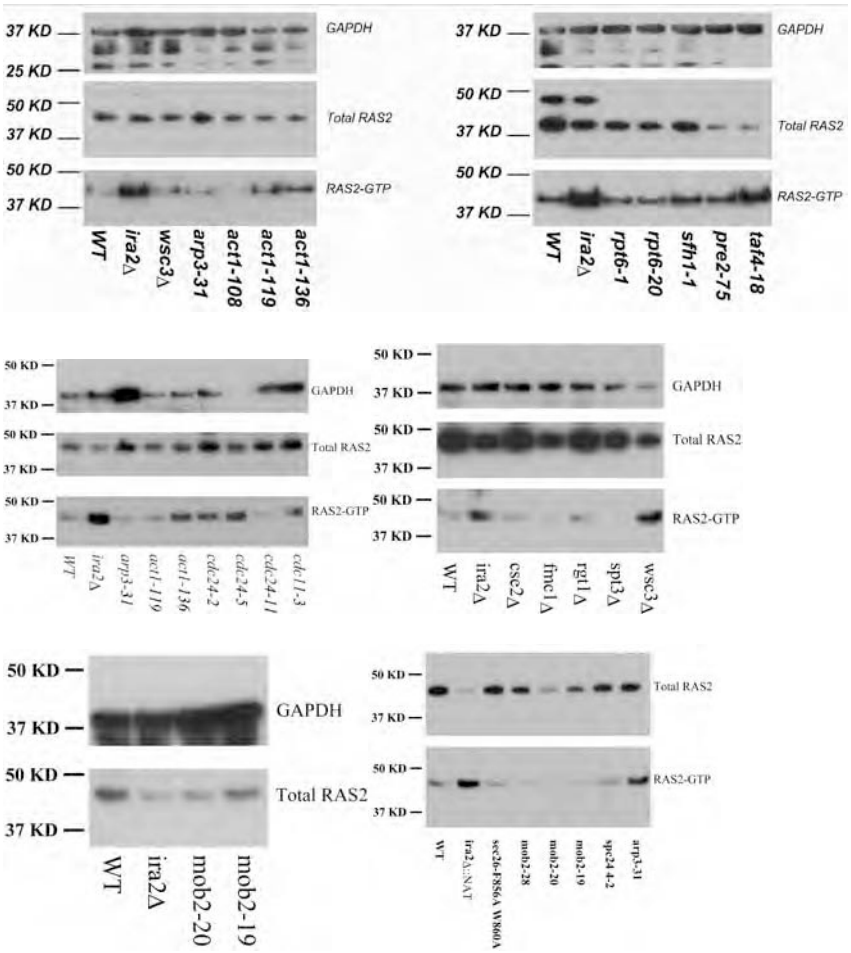

Figure. 3. Examples of Ras activation assays used to define how the putative Ira1- and Ira2-interacting genes affect Ras signaling. Ras activity is assessed by determing the amount of GTP-bound (active Ras) compared to total Ras. GTP-bound Ras is specifically isolated by incubation with agarose beads containing the Ras-GTP-binding domain of Raf. Following pull-down, proteins are detected by immunoblotting. A portion of the cell lysate before incubation with Raf agarose beads is used to measure total Ras.

It has been reported that Wsc3 is involved in the Ras signaling pathway but it is not clear whether it works upstream or downstream of Ras [1]. Based on our Ras pull-down assay result, we propose that Wsc3 could function upstream of Ras2 and regulate cellular Ras2-GTP content. It has been reported that overexpression of *IRA2* can suppress the heat shock sensitivity of the *wsc3*Δ mutant, and deletion of *RAS2* rescues the heat shock sensitive phenotype of the *wsc3*Δ strain [1]. Our current working model is that WSC3 might work through activating the function of IRA1 or suppressing a Ras-GEF (for example, CDC25 or SDC25). Further detailed analysis on these proteins in wsc3Δ cells will be needed to validate this model and will be carried out in the future years of funding for this project.

Our data show that

arp3-31, act 1-119, act1-136, cdc24-2, and *cdc24-5* have slightly higher Ras2-GTP content and we have repeated the pull-down assay and confirmed that these strains do indeed have higher Ras-GTP content. All these protein are well known to be involved in actin filament organization. These data suggest that there is a connection between Ras activation and actin filament organization. In the TS library screen, multiple act1 TS mutants (act1-108, actin1-119, act1-122, act1-25, act1-136) and cdc24 TS mutant (cdc24-1, cdc24-2, cdc24-5, cdc24-3, cdc24-11) all showed strong alleviating genetic interaction with ira1 or ira2 deletion strains. A connection between NF1 and actin is completely novel and unexpected and underscores the power of the yeast system to uncover new and unexpected pathways involved in NF1 biology. How these two biological processes are connected and how actin filament organization affects Ras-GTP levels needs to be explored with more detailed assays. We will pursue these in the next years of funding for this project.

Currently, our knowledge about Ras proteins is that they can switch between GDP bound inactive form and GTP bound active form, and therefore serve as a molecular switch to transduction signaling. During our Ras pull-down assay, we noticed that, in saturated cultures, the ira1 and ira2 deletion strains exhibited dramatically lower total Ras2 protein levels compared with WT strain, despite having elevated Ras2-GTP levels. We confirmed these observations with multiple experiments with *ira2*Δ cells in different pull-down conditions (Fig. 3). We have further confirmed the equal loading of total protein in these samples by checking the GAPDH protein levels. How do ira1 and ira2 regulate RAS2 protein level? What is the biological consequences of down-regulating Ras2 protein levels? Interestingly, although wsc3 deletion, arp3-31, act1-119, act1-136, cdc24-2, and cdc24-5 strains also affect Ras2-GTP content, they do not affect total Ras2 protein levels. On the other hand, other hits do affect Ras2 protein level but not Ras2-GTP content (for example, mob2-19 and mob2-20). Do they affect Ras2 protein levels through the same mechanisms of ira1 and ira2? Or do they act through transcription and/or translational regulation? Or even post-translation modification? Recently, it has been reported that H-Ras is degraded by Wnt/β-catenin signaling via β-TrCP-mediated polyubiquitylation. Is that the only pathway or there are multiple level regulations on RAS protein level? In our SGA screen hits, there are many hits that are known to be involved in transcription regulation, mRNA processing, translation regulation, or post-translation modification, and protein degradation. Do these hits also affect Ras protein level? We found that a strain with a mutation in one of the subunits of the proteasome, pre2-75, has lower Ras2 protein level but similar amounts of Ras-GTP compared with WT. Another gene mutant taf4-18, which was known to be involved in RNA polymerase II transcription initiation, shows both lower Ras2 protein level and higher RAS-GTP content compare with WT. Obvious, more detailed analyze of Ras2 mRNA level, protein level and protein modification in these hits strains will be needed in order to answer these questions.

We are eager to continue to pursue the results from the first year of funding for this project. Our top priority will be to finish the remaining Ras-GTP pull-down assay for the iodine staining positive hits. In light of our observation of varying levels of total Ras protein in different strain backgrounds, we will also systematically check Ras protein level in all hits by western blot. To define how Ras levels are affected, we will assess Ras2 mRNA level by RT-PCR and Ras2 protein stability by immunoblotting. We have laid the groundwork for year 2 of the project, where we will take some of our best candidates modifier genes and test for physical interaction with IRA1 and/or IRA2 by TAP-tag purification as well as co-immunoprecipitation.

Key Research Accomplishments

- Discovered a previously unappreciated connection between actin, ras signaling, and potentially NF1.
- Discovered a new level of Ras regulatory complexity (protein levels vs. GTP-bound state).
- Developed a high-confidence set of ras-dependent and ras-independent candiate NF1 modifier genes that will aid future functional characterization as well as validate in animal models.

Reportable Outcomes

- Undergraduate researcher Zinaida Dedeic presented a poster describing her research on this project at the University of Pennsylvania Department of Cell and Developmental Biology Annual Symposium.
- Zinaida Dedeic successfully accepted into University of Chicago Ph.D. program based on her successful research experiences and training supported by this grant.

Conclusion

In year 1 of our project we have defined a set of yeast genes that interact genetically with the NF1 homologs, *IRA1* and *IRA2*, and have gone on to functionally categorize these genes as being either ras-dependent or ras-independent. These data will facilitate our continued mechanistic experiments aimed at identifying novel functions for Neurofibromin as well as defining novel regulators of its known function as a regulator of the ras signaling pathway.

References

1. Verna, J., et al., *A family of genes required for maintenance of cell wall integrity and for the stress response in Saccharomyces cerevisiae.* Proc Natl Acad Sci U S A, 1997. **94**(25): p. 13804-9.

Appendices

None